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GRANT NUMBER DAMD17-94-J-4287

TITLE: Analysis of the Regulation of Expression of Transforming Growth Factor-Beta in Human Breast Cancer Cells

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REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1997	Annual (1 Oct 96 - 30 Sep 97)

4. TITLE AND SUBTITLE
Analysis of the Regulation of Expression of Transforming Growth Factor-Beta in Human Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4287

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

This grant entails two objectives. The first objective derived from prior work which identified TGF-β1 as an important cytokine in breast cancer. The specific goal of this objective was to identify the molecular mechanism of overexpression of TGF-β1. We have analyzed paired normal/tumor specimens from patients and have determined that this genetic locus is not amplified in a significant proportion of patients. In other work we have recently obtained data which refutes the suggestion that overexpression of TGF-β1 is associated with poor patient outcome. The second objective of the grant was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cell lines. In the past year we have focussed on examination of the methylation pattern at CpG sites in the vicinity of the two TGF-β3 promoters. We have observed that whereas CpG sites closest to the breast cancer-specific downstream promoter are methylated in non breast cancer cells, they are unmethylated in breast cancer cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Gene Expression, Growth Factors, TGF-Beta, Promoter Analysis, Estrogen, DNA-Protein Interactions, Humans, Anatomical Samples, Breast Cancer	15
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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PI - Signature 10/29/97 Date

ANNUAL REPORT
Grant Number DAMD17-J-4287
PI: Bradley A. Arrick, M.D., Ph.D.
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Introduction

This grant covers two distinct objectives, both of which derived from prior work by ourselves and others which have identified TGF-β as a potentially important cytokine in the biology of breast cancer. For established tumors, overexpression of TGF-β may result in increased *in vivo* tumor growth and metastatic spread (Gorsch et al. 1992). The first objective, as outlined in the initial application, involved the analysis of resected breast cancer specimens, with the main goal of determining whether overproduction of TGF-β in certain tumors is due to gene amplification (a common genetic alteration in tumor tissue) or some other mechanism, such as increased mRNA expression. The second objective of the grant was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cells. These studies stem from our prior work in which we have characterized an alternative downstream TGF-β3 promoter which seems to be functional only in breast cancer cells (Arrick et al. 1994).

Body of Report

First Objective (Objective A)

Amplification of the TGF-β1 gene locus in human breast cancer tissue was evaluated by the method of differential polymerase chain reaction (PCR). This method involves the co-amplification of the locus in question, with gene-specific primers, along with a reference gene, with its specific primers, in the same reaction tube. Total genomic DNA is used as template. If the target gene is present in greater copy number than the reference gene, its PCR product will be generated in greater amounts and its amplification will suppress the amplification of the reference gene. As described by the developers of this method (Frye et al. 1989, Neubauer et al. 1992), the PCR products are separated by electrophoresis thru a polyacrylamide gel, and the relative intensities of the bands representing amplification of target and reference gene are quantitated, serving as a measure of gene copy number for the target gene.

We have prepared genomic DNA from paired specimens of breast cancer and normal breast tissue from patients who have undergone resection at our institution. Specimens were stored at -70°C until processing, which consisted of grinding with morter and pestle under liquid nitrogen until a powder resulted. This was then added to 4 ml of

100 mM NaCl, 10 mM Tris pH8.0, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K, and then placed at 50°C for overnight digestion. The next day the solution was extracted with phenol chloroform, and the DNA precipiated with ethanol, resuspended in TE and quantitated by uv absorbtion.

For PCR amplification of our gene, we have used primers for TGF-β1 which amplify a 131 bp fragment. For the reference gene to be co-amplified in our competitive PCR we have used the interferon-β gene (IFN-β), employing primers which amplify a 119 bp fragment. Specifics of primers, PCR conditions, and analysis of specimens follows:

Amplimers:
TGF-β-Forward (5'-primer): CGG CAG CTG TAC ATT GAC TT (5' position 1739)
TGF-β-Reverse (3'-primer): ACC TTG CTG TAC TGC GTG TC (3' position 1850)
Optimal annealing temperature: 55.7°C
Product length: 131 bp

IFN-β-Forward (5'-primer): GTG TCT CCT CCA AAT TGC TC (5' position 12)
IFN-β-Reverse (3'-primer): GCC ACA GGA GCT TCT GAC AC (3' position 111)
(Ref: Ohno & Taniguchi, 1982)

PCR conditions: For each 50-µl PCR reaction, we used 400 to 800 ng of genomic DNA as template. PCR conditions are as following: 1.5 mM $MgCl_2$, 0.2 mM dNTPs , 75 picomoles of each of the oligonucleotide primers amplifying TGF-β, 50 picomoles each of oligonucleotide primers amplifying IFN-β, and a standard GeneAmp buffer II (Perkin Elmer) at 1X. Reactions were overlayed with oil, and PCR cycle conditions as follows: first 96°C for 10 min; then 85°C for 3 min while portion of reaction d_2H_2O combined with 0.5 µl of AmpliTaq Polymerase (Perkin Elmer) is added (for hot start), then 36 cycles with an annealing temperature of 50°C (40 sec), extension temperature of 72°C (1 min), melting at 95°C (30 sec). For every set of PCR reactions, we include a no-DNA reaction tube as negative control. For each case, tumor DNA and normal breast DNA are used as template in parallel.

Electrophoresis: To achieve resoluton of the expected 119- and 131-bp bands, we ran 30µl of PCR product on 12% polyacrylamide non-denaturing gel at 28 mAmp for 3 hrs, then stained with ethidium bromide. The gels were photographed with Polaroid type 665 positive/negative film, and the resulting images on the negative film quantitated as below.

Scanning/Quantification: Using Silver Scan III, we scanned in the images from the negative film, zooming in specifically between 100 bp to 200 bp. In each reaction lane, we quantify the intensity of the amplified bands of 119 bp and 131 bp by drawing a same-sized box around each band, plus an empty box (in the same lane) to serve as a measure of the background. The software IPLabGel (Molecular Dynamics) was then used to calculate the pixel density within each box (see fig 1).

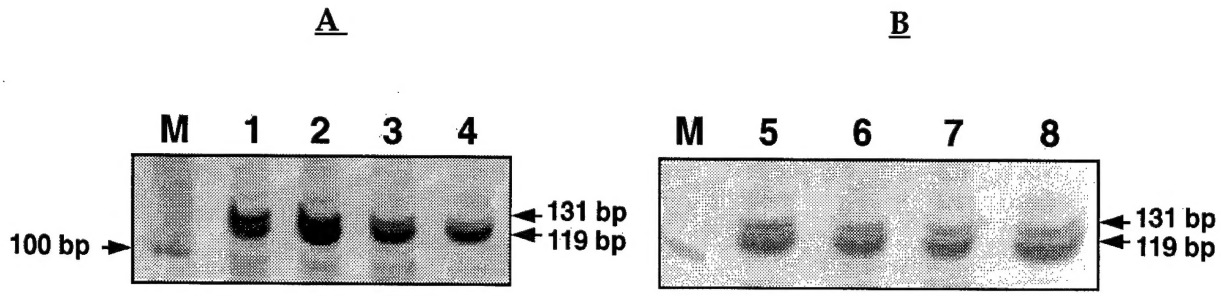


Fig 1. Portions of the 12% polyacrylamide gels loaded with 30 µl of the 50-µl amplification reactions. M = 100-bp ladder. Lanes 1, 2 = normal and tumor DNA of patient #1, respectively; lanes 3, 4 = normal and tumor DNA of patient #2, respectively; lanes 5, 6 = normal and tumor DNA of patient #3, respectively; and lanes 7, 8 = normal and tumor DNA of patient #4, respectively. These are representative gels, from which the band intensities were quantitated by scanning.

The calculations for determination of gene amplification were as followings:

a= pixel count of 119 bp IFN-β-derived band
b= pixel count of 131 bp TGF-β-derived band
x= pixel count of background within the same reaction

then, Y = (b-x)/(a-x) serves as a measure of the amplification of the TGF-β gene normalized to the amplification of the reference gene IFN-β.

Results:
Seventeen paired specimens were analyzed, of which fifteen yielded PCR products from which quantitation was possible. Samples are labeled #-1 for normal tissue and #-2 for tumor tissue from the same patient. The ratio of Y values obtained from normal vs tumor thus represents a measure of gene amplification in the tumor.

Table I contains the data, listing the paired specimens, the calculated Y values, followed by the ratio, with the value from the normal tissue adjusted to be one.

TABLE I

Specimen #s	Y value	Normal vs Tumor Ratio
1-1	.32	
1-2	.63	1 : 2
2-1	.34	
2-2	.51	1 : 1.5
4-1	1.0	
4-2	.67	1 : 0.67
5-1	.80	
5-2	.56	1 : 0.7
6-1	.75	
6-2	.75	1 : 1
7-1	.78	
7-2	.62	1 : 0.8
8-1	.65	
8-2	.80	1 : 1.2
9-1	.68	
9-2	.63	1 : 0.9
11-1	.62	
11-2	.55	1 : 0.9
12-1	.75	
12-2	.67	1 : 0.9
13-1	.36	
13-2	.38	1 : 1.05
14-1	.81	
14-2	.66	1 : 0.8
15-1	.70	
15-2	.70	1 : 1
16-1	.73	
16-2	.33	1 : 0.45
17-1	.54	
17-2	.33	1 : 0.6

Interpretation of results:
Using a greater-than-2-fold amplification cut off in the ratio of normal to tumor DNA for the designation of gene amplification, none of the 15 breast cancer cases from which data were obtained demonstrated amplification of the TGF-β1 gene. We can use statistical analysis to obtain an estimate of incidence of TGF-β amplification in breast cancer. In general, amplification of genetic loci which are of clinical or biological significance (and thus worthy of further investigation) are evident in over 20% of cases. In other words, amplification which is found in less than 20% of cases is unlikely to be an important feature of the disease process. Observing no cases of amplification after examination of 15 cases means that there is a greater than 95% chance that less than 20% of breast cancers have amplification of this locus (Zelen 1993).

As discussed in the "Conclusions" section, in other studies (not funded by this grant but concluded within the past year) we have analyzed a large set of patient specimens for TGF-β expression and found no correlation between intensity of staining and patient outcome. Given that the underlying hypothesis that TGF-β expression is an important determinant of outcome has been negated by our recent analyses, there is little justification for proceeding with analysis of mRNA levels in resected specimens.

Second Objective
The entirety of this objective is to understand the molecular basis by which breast cancer cells, unlike all other cell types examined by ourselves and others, utilize two promoters for transcription of the TGF-β3 gene, one the previously characterized promoter and the other approximately 860 nucleotides downstream, still within the 5' noncoding region of the gene (Arrick et al., 1994).

Objectives B-1 and B-2

As detailed in our previous reports, we have in this year of the grant focussed efforts on the analysis of CpG methylation at the TGF-β3 promoter. Last year we included in our report data in which specific CpG sites contained within restriction endonuclease sites (enzymes either sensitive to methylation or not) were analyzed via southern analysis. In that report we discussed the reasons for a more detailed mapping of the methylation pattern of all CpGs in this genomic region, not just those few which are a part of such restriction enzyme recognition sites. The return to analysis of DNase hypersensitivity sites has been postponed until areas of different methylation (comparing breast and nonbreast cells) are identified.

For mapping of CpG methylation, we have utilized the bisulfite conversion reaction. This involves first isolation of genomic DNA from the cell lines, followed by alkaline denaturation. DNA (now single stranded) is then reacted with sodium bisulfite under conditions in which nonmethylated cytosines are converted to uracil bases but not so harsh as to fully convert all cytosines (methylated or not). Primers which would amplify bisulfite-converted DNA are then used to amplify sections of genomic DNA, and the resulting PCR products analyzed by sequence analysis. When a position

known to contain a CpG in the starting DNA is converted to TpG we conclude that that cytosine was not methylated. In preliminary experiments, we adjusted the reaction conditions of the bisulfite conversion such that just under 100% of the unmethylated cytosines were converted. This is because more aggressive conditions (time, temperature, etc) which would guarantee 100% conversion of unmethylated cytosines also resulted in some conversion of methylated cytosines (as well as extensive DNA degradation). Therefore, we have numerically adjusted our estimates of the methylation status at particular CpG sites by a factor which takes into account the efficiency of bisulfite conversion as determined by examination of the conversion rate of the non-CpG sites in the adjacent sequences(assuming that all non-CpG sites are not methylated).

Method of bisulfite conversion.

Genomic DNA was isolated from tissue culture cells using the Puregene DNA Isolation kit from Gentra Systems, Inc. The T47-D and SK-BR3 cell lines serve as our breast cancer cells, and the HT-1080 cell line is our non-breast cancer cell line (all express TGF-β3). Each genomic DNA conversion reaction was done twice, with samples from the above lines processed in parallel. First, DNA was digested overnight at 37°C with BglII (20U for 10 μg of DNA), followed by ethanol precipitation and resuspension in water. Denaturation then followed by a 15 min treatment with 0.3 M NaOH. For each reaction, fresh solutions of 5.5 M sodium bisulfite ($NaHSO_3$) and 110 mM hydroquinone, pH5, were prepared and kept in the dark until use. This solution was added to the DNA/NaOH solution (ratio 9:1), the mixture overlayed with mineral oil, and then incubated at 55°C in the dark for 4 hrs. The DNA was then desalted (using "Wizard DNA resin" from Promega), and precipitated. The previously unmodified cytosines at this stage are sulfonated uracils. Desulfation proceeded by base treatment (0.3 M NaOH, 15 min, 37°C), followed by neutralization and precipitation. The resulting DNA was then resuspended in 50 μl of Tris-EDTA. The specific conditions of this method is an adaptation of published methods (Frommer et al. 1992 and Raizis et al, 1995).

PCR amplification of bisulfite-converted DNA

The following criteria was consider in designing the primers. (A) Because the bisulfite conversion changes the strands so they are no longer complementary, the primers have to be be designed in a strand specific manner. (B) Primers were to be somewhat longer in length, i.e. from 28-31 bases, in order to achieve optimal annealing temperatures with the reduced GC content. (C) Positions targeted within the sequence that were initially cytosines within a CpH site would be assumed to be converted to thymines, and therefore the appropriate base would be used (thymine in the upper primers and adenine in the lower primers. (D) In postions that were CpG sites in the target sequence an equal mix of the appropriate bases (C and T in the upper primers, G and A in the lower primers) would be used so that the site would still be targeted regardless of methylation. (E) CpG sites would be avoided in the 3' end of the primer since this is the most important region of primer annealing to template. We have used nested PCR, first amplifying a 2.2 kb region going from approximately 1.2 kb upstream of P1 (the 5'-most promoter common to both breast cancer and non breast cancer cells) to approximately 120 nucleotides downstream of the P2 transcription initiation site (the downstream

breast cancer specific promoter). The resulting PCR reaction product was then used as template for PCR utilizing the internal primers, dividing the region into three overlapping segments.

For the first PCR, 2 µl of converted genomic DNA was added to 38 µL of buffer [1x final concentration - 200mM Tris-HCl pH 8.4, 500mM KCl], dNTP's (Promega) [2.0 mM each final concentration], $MgCl_2$ (Perkin Elmer) [2.5mM final concentration], and 0.2 µM upper primer 992 (see below for primer sequences). Afterwards, a mineral oil overlay was added. For this PCR reaction a hot start was used: the tubes were first heated to 90°C for up to ten minutes in the thermocycler, then 10 µl containing 2.5 U Taq polymerase and 0.2 µM of lower primer 3155 was added. The reaction was then heated to 95°C for 3 minutes. Then the reaction went through 38 cycles of 60 seconds at 95°C, 90 seconds at 54°C, and 3 minutes at 72°C. After these cycles were completed, a final extension for 6 minutes at 72°C was performed. The reaction soaked at 4°C until frozen in a -20°C freezer. An aliquot from this reaction was then used as template in a subsqeunt nested PCR. A pair of internal primers were used to amplify an upstream portion (upper primer 1091 and lower primer 1855), a midstream portion (upper primer 1678 and lower primer 2631), or a downstream portion (upper primer 2334 and lower primer 3104). 25 µl reactions were prepared on ice containing 1 µL of the initial PCR reaction diluted 1:25 by adding it to 24 µl of buffer [1x], dNTP's [2.0 mM each], $MgCl_2$ [2.5 mM], primer pair [0.5 µM each] and Taq [1.25 U]. The complete reaction was heated to 95°C for 3 minutes, then carried through 42 cycles of 60 seconds at 95°C, 90 seconds at 54°C and 90 seconds at 72°C. The resulting amplification products were viewed by electrophresis on a 1% argose (Gibco) TBE gel, stained with ethidium bromide. The expected product was subcloned by TA cloning (Promega). Multiple clones were then grown up, plasmid DNA containing the PCR products purified, and the PCR products sequenced by dideoxy sequencing, using primers from the plasmid backbone itself extending into the cloned portion.

Primer sequences for first round of PCR:

upper "992": ATT CGT AAA AGT GAT TTA TCG TTG TGT T
lower "3155": ACC TCC CCA AAT CCC AAA AAC TAA AAC T

Primer sequences for nested PCR:

upper "1091": GAG TGA GAT GGG GTG GAG CGG TAT TTA TTT
lower "1855": CGT CCG ACC CGA TCT ACT CTC CCT CCT AAT

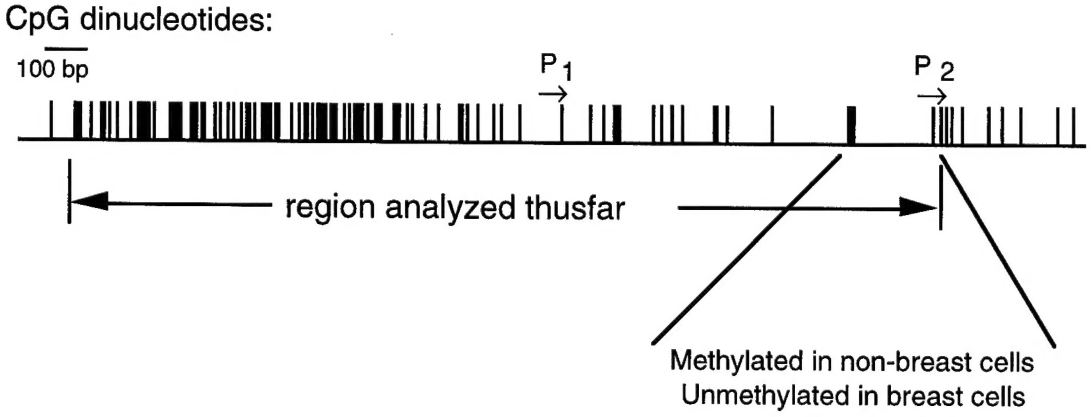
upper "1678": TTT TGT TAA TGA AGA ATC GGG TTA GGA T
lower "2631": CCA TTC ATA CTT TCT CTT TTA TTT ACA CTT

upper "2334": GGA AGA GGC GTG CGA GAG AAG GAA TAA T
lower "3104": CCA AAA AAC GCT AAC CCT AAA AAC GAA A

Results of methylation analyses

Figure 2 displays the CpG sites at this genetic locus, relative to the two promoters under study. We have observed no differences in CpG methylation in the majority of sites, comparing the DNA obtained from the HT1080 cells with that from the T47-D and SKBR3 cells, with the exception of the most downstream, P2-proximal region. Three CpGs in close promiximity to eachother, located about 170 bp upstream of P2's transcription initiation site (represented as a thick band in Fig 2 because of the closeness of the sites) were 90-100% methylated in the HT1080 cells but were found to be <20% methylated in the T47D and SKBR3 cells. The actual sequence in which these CpGs are found is as follows: TCGCCAAGCGGCGTTT. In addition, two CpGs within 50 bp of the transcription initiation site of P2 were found to be differentially methylated, as above. These differences in methylation are consistent with the hypothesis that certain CpG sites (presumably with a functional relation to P2 utilization) will be unmethylated in the breast cancer cells.

Fig. 2



Objectives B-3 and B-4

The experiments for Objective B-3 and B-4 relate to the transfection of cells with chimeric TGF-β3 promoter constructs and the subsequent utilization of those cells for the elucidation of the promoter element(s) involved in the downregulation of this gene by estrogen. Critical to this effort is the successful creation of stably transfected estrogen receptor-positive cells with a chimeric promoter construct which demonstrates transcription initiation at both promoters. In last year's report, we reported success in this regard only with the SKBR3 line (which does not contain the estrogen receptor). In subsequent transfection attempts, we have been unable to generate the required cells in a line which is estrogen-receptor positive (T47-D). Transient transfections of T47-D cells with the chloramphenicol acetyltransferase (CAT)-chimeric promoter plasmid used in Figure 6 of the original proposal (which demostrated promoter activity in SKBR3 cells but not A673 and HT1080 cells, both of which are non-breast in origin) were performed. Unexpectedly, the T47-D cells yielded the same result as the HT1080 and A673 cells and

did not show the promoter activity that was evident in the SKBR3 cells. This may explain our inability to obtain stable transfectants of cells with the neo resistance gene driven by this presumptive promoter sequence in T47D cells.

Proposed modifications in Statement of Work for the final year of funding

With regard to the analysis of mechanisms of TGF-β overexpression in breast cancers, we have determined that gene amplification is not a feature of breast cancer. Furthermore, in other work completed this past year, we have found that our initial observation of an association between clinical outcome and TGF-β immunohistochemical intensity did not hold up upon examination of a larger, more homogeneous patient population. This being the case, there is little justification for pursuing this issue. Specifically, we feel it would be a waste of effort to analyze mRNA levels in the resected specimens.

Clearly, with regard to our studies to analyze the molecular basis of promoter utilization for TGF-β3, we have had considerable difficulty keeping pace with the initial statement of work for this grant proposal. Our work to date has suggested differences in methylation only at the CpGs closest to the downstream promoter. We intend to focus efforts in the remaining year on this finding. We need to extend our analysis of CpG methylation further downstream (at least a few hundred bases). This region will then be examined for DNase hypersensitivity sites. We will also test the hypothesis that demethylation of this region in the nonbreast cancer cells can activate the downstream promoter using azacytidine.

Conclusions

With regard to the first objective, we can conclude that the TGF-β1 genetic locus is not amplified in breast cancer. Other studies we have undertaken, not funded by this grant, in which tissue specimens from a large homogeneous population of breast cancer patients were studied by immunohistochemistry have indicated that there is no association between tumor cell TGF-β expression and clinical outcome.

In our analysis of the CpG methylation status at the TGF-β3 promoter we have observed a marked difference in methylation at the CpG sites in closest proximity to the downstream promoter. Specifically, DNA from the two breast cancer cell lines (in which the downstream promoter is active) demonstrated minimal methylation at these CpG sites, whereas DNA from the non breast cancer cell line (HT1080) was completely methylated at these sites. This is consistent with, and may be the underlying basis for, the differential utilization of the downstream promoter. We are currently expanding our analysis of methylation to more of the near-by CpG sites in the vicinity of the downstream promoter.

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